

Effect on Pulp Healing of CO₂ Laser Irradiation and Direct Pulp Capping with Experimentally Developed Adhesive Resin Systems Containing Reparative Dentin-promoting Agents

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Purpose: The purpose of this study was to examine the reaction and hard tissue derivation of rat pulp directly capped with experimentally developed adhesive resin systems after CO₂ laser irradiation.

Materials and Methods: The experimentally developed bonding agents used for direct pulp capping contained four kinds of calcium phosphate: hydroxyapatite, dicalcium phosphate dehydrate, β-tricalcium phosphate, and octacalcium phosphate, as well as Mega Bond (MB) primer (MBP) used for adhesive treatment. The intensities of irradiation of the CO₂ laser were set at three stages: low, medium (standard) with LLLT action, and high. The 12 experimental groups were formed by combining the three laser groups with four kinds of experimentally developed bonding agents. MBP was applied to the control group after direct pulp capping with Dycal without laser irradiation. In all groups, the cavities were filled with Clearfil AP-X and photopolymerized. Histopathological and immunohistochemical examinations were undertaken 3, 7, and 14 days after direct pulp capping.

Results: There was no significant difference in the wound healing of exposed pulp among the laser-irradiated experimental groups. However, the group irradiated with the low-intensity laser showed faster pulp healing and dentin bridge formation than the other laser groups.

Conclusion: There was no significant difference in wound healing of exposed pulp between each experimental group and the control group. It was suggested that low irradiation condition of CO₂ laser and direct pulp capping with experimentally developed bonding agents containing calcium phosphate were comparable to the preparation of calcium hydroxide DY. The thickness of irritative dentin formed on the pulpal wall showed a tendency to increase as the intensity of irradiation was raised.

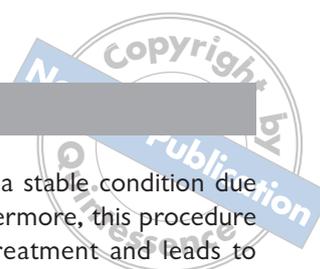
Keywords: direct pulp capping, experimental adhesive resin system, reparative dentin-promoting agent, CO₂ laser, rat pulp, dentin bridge.

J Oral Laser Application 2008; 8: 257-273.

Submitted for publication: 10.09.08; accepted for publication: 16.10.08.

Since the CO₂ laser was developed by Patel in 1964,¹ it has been broadly used as an energy efficient laser. Nowadays, the CO₂ laser is applied in various fields of

dental treatment such as caries prevention, coronal restoration, endodontic treatment, periodontal treatment, oral surgery, dental anesthesia, and so on.



Sometimes we encounter accidental dental pulp exposure in clinical practice. Several studies have reported that laser irradiation was effective for hemostasis, coagulation and sterilization of the surface of exposed pulp, and reparative dentin formation.²⁻⁸ On the other hand, it was reported that the denaturation layer yielded by laser irradiation caused a delay in pulp wound healing.⁹ Thus, there are conflicting views regarding the effect of laser irradiation on the wound healing process of exposed pulp and dentin bridge formation.

It has been observed that most of the energy of the CO₂ laser is absorbed within a depth of 0.1 to 0.2 mm of the top layer of the soft tissue and the heat denaturation layer yielded by CO₂ laser irradiation is less than 0.5 mm thick. Therefore, the use of the CO₂ laser is suitable for arresting hemorrhage from exposed pulp because of heat diffusion to the subsurface of pulp tissue. However, we speculate that the effect of CO₂ laser irradiation on the exposed pulp depends upon the intensity of laser irradiation.

Calcium hydroxide¹⁰ and its preparations^{11,12} have been generally used for the treatment of pulp exposure for a long time, because the strong alkalinity of calcium hydroxide is effective in encouraging the formation of a dentin bridge.¹³⁻¹⁵ However, they contain a weak point in that they tend to produce a necrosis layer 0.5 to 1.0 mm thick on the surface of an exposed pulp.¹⁶ Recently, calcium phosphate compounds such as hydroxyapatite (HAP),¹⁷⁻¹⁹ dicalcium phosphate dihydrate (DCPD, brushite),²⁰ tricalcium phosphate (α -TCP, β -TCP, whitlockite),^{19,21-27} tetracalcium phosphate,²⁸⁻³⁰ and octacalcium phosphate (OCP)^{27,31-35} have become available for direct pulp capping. DCPD, α -TCP, β -TCP and OCP change to HAP when mixed with an aqueous solution such as water.^{20,24,29-31} Several studies have reported that a dentin bridge was formed on the surface of exposed pulp without formation of a necrotic layer when calcium phosphate was applied to the exposed pulp.^{24,29} However, there is something left to be desired regarding the mechanical properties of solid calcium phosphate, but problems have to be solved related to the setting manner and operation.²⁰ When we apply the calcium phosphate powder mixed with water to the exposed pulp, blood exudation considerably affects various properties of the mixture because of its slow setting. Therefore, we have developed various kinds of experimental bonding agents with the addition of calcium phosphate, and they have been put on the market by Kuraray Medical as direct pulp-capping agents. This experimental bonding agent used with adhesive resin systems is expected to prevent bacterial in-

fection and to keep the pulp in a stable condition due to excellent cavity sealing. Furthermore, this procedure simplifies direct pulp-capping treatment and leads to shorter operation time.

We previously reported the efficacy of resinous materials in direct pulp-capping treatment using monkey pulp.³⁶⁻⁴⁰ More recently, we also investigated the effect of laser irradiation on direct pulp capping.⁴¹⁻⁴⁴ The results showed that laser irradiation was effective in arresting hemorrhaging from the exposed pulp, but delayed the wound healing of pulp tissue, and revealed the problems in the intensity levels of laser irradiation and types of laser apparatus.

Therefore, the present study examined the effects of the intensity parameters of CO₂ laser irradiation on direct pulp capping, and the effects of stable carbonized layers and heat denaturation layers produced by laser irradiation on pulp wound healing. In the pilot study, we applied various intensities of CO₂ laser irradiation to exposed rat pulp, and evaluated the thickness of carbonization layers and heat denaturation layers on the exposure surface of the rat pulp. From the results of the pilot study, we set three intensities of CO₂ laser irradiation: low, medium with LLLT (low level laser therapy), and high to irradiate the exposed pulp.

Suzuki et al⁴⁴ reported that experimental adhesive systems formed by combining 5-NMSA and HAP were effective in initiating an early repair process after direct pulp capping. On the basis of that study, we have developed four kinds of experimental bonding agents made by the combination of experimental monomers and varying amounts of calcium phosphate powder. Each amount of calcium phosphate powder was prepared by compounding HAP with brushite, whitlockite and OCP at set ratios. In this study, the exposed rat pulp was directly capped with these newly developed bonding agents after CO₂ laser irradiation, and morphological changes and the ability of pulp tissue to form hard tissue was evaluated histopathologically and immunohistochemically.

MATERIALS AND METHODS

Experimental Animals

Before the experiments, approval was obtained from the Laboratory Animal Committee of the School of Life Dentistry at Niigata, The Nippon Dental University.

Seventy-five rats (Sprague-Dawley male, 6 weeks old and about 180 g in weight) were used. The rats were fed solid food MF (Oriental Yeast; Tokyo, Japan)

Table 1 Materials

Materials	Abr.	Lot #	Composition	Manufacturer
MegaBond Primer	MBP	00518A	2-hydroxyethyl methacrylate, hydrophilic dimethacrylate, 10-methacryloyloxydecyl dihydrogen phosphate, N,N-diethanol-p-toluidine, d,l-camphorquinone, water	Kuraray Medical
MegaBond Bonding Agent	MBB	0373AA	2-hydroxyethyl methacrylate, 10-methacryloyloxydecyl dihydrogen phosphate (MDP), amine, d,l-camphorquinone, hydrophobic aliphatic dimethacrylate, silanated colloidal silica, bisphenol A diglycidylmethacrylate	Kuraray
Experimental Bonding Monomer	EBM	041110	Similar composition to MBB	
Experimental Bonding Agents	MB8	041006	MBB containing 5 wt% hydroxyapatite and 5 wt% brushite	Kuraray
	MB9	041006	MBB containing 5 wt% hydroxyapatite and 5 wt% whitlockite	
	MB11	041006	MBB containing 4 wt% hydroxyapatite, 3 wt% brushite and 3 wt% whitlockite	
	MB12	041006	MBB containing 4 wt% hydroxyapatite, 2 wt% brushite, 2 wt% whitlockite and 2 wt% octacalcium phosphate	
Dycal	DY	020121	Base paste: ester glycol salicylate, calcium phosphate, Ca tungstate, ZnO	Dentsply Caulk
		030729	Catalyst paste: ethylene toluene sulfon amide, Ca(OH) ₂ , ZnO, Ti ₂ O ₃ , Zn stearate	
Clearfil AP-X (A3)	AP-X	00898A	Bis-GMA, TEG-DMA, barium glass, silanated colloidal silica, d,l-camphorquinone	Kuraray Medical

and water for 2 to 3 weeks in the cages of the breeding house affiliated with our university. A total of 288 noncarious teeth – maxillary first and second molars – was treated by direct pulp capping when the rodents were 8 to 9 weeks old and weighed 280 to 340 g.

CO₂ Laser

A CO₂ laser device (Opelaser 03S II SP, Lot #MD-036, Yoshida Dental; Tokyo, Japan) was used. The specifications of this CO₂ laser are as follows: wavelength, 10.6 μm; power output, 0.5 to 5.0 W (adjustable per 0.1 W); focus depth, 0.1 to 0.2 mm; focus beam diameter, 0.4 mm; and green guide beam wavelength, 532 nm. Different power modes were available, such as continuous wave mode and super-pulsed modes 1 and 2. The options of exposure modes included continuous irradiation, super-pulsed irradiation (a cycle of 0.001 to 0.9 s) and repeat pulse irradiation modes. The W type (60-degree head) handpiece was used in this study.

Materials

The experimental materials used in this study are shown in Table 1. Mega Bond (MB) Primer (MBP, Lot #00518A, Kuraray Medical; Tokyo, Japan) was used as the primer, four kinds of experimentally developed bonding agents (MB8, MB9, MB11 and MB12, Lot #041006, Kuraray Medical) were used for the laser irradiation groups as direct-pulp capping materials, and Dycal (DY, Lot #020121, Lot #030729, Dentsply Caulk; Milford, DE, USA) with MB was used for the control group as the direct-pulp capping material. Clearfil AP-X (AP-X, Lot #00898A, Kuraray Medical) was used as the restorative resin composite. The composition of each experimental bonding agent is shown in Table 1. MB8 was an experimental bonding monomer containing 5 wt% HAP and 5 wt% brushite. MB9 was an experimental bonding monomer containing 5 wt% HAP and 5 wt% whitlockite. MB11 was an experimental bonding monomer containing 4 wt% HAP, 3 wt% brushite, and 3 wt% whitlockite. MB12 was an experimental bonding monomer containing 4 wt% HAP, 2 wt% brushite, 2 wt% whitlockite, and 2 wt% OCP.

Table 2 Experimental groups

Groups	Materials used	CO ₂ laser irradiation parameters
1	MBP+MB8	Low intensity: 0.5 W, SP2 mode (repeatedly, irradiation 1 ms/stop 10 ms), defocus, irradiation time: 3 s, irradiation distance: 2 cm
2	MBP+MB9	
3	MBP+MB11	
4	MBP+MB12	
5	MBP+MB8	Medium (standard) intensity with LLLT Medium intensity: 0.5 W, SP1 mode (repeatedly, irradiation 10 ms/stop 10 ms), defocus, irradiation time: 3 s, irradiation distance: 2 cm LLLT: 0.5 W, CW mode (continuously), defocus, irradiation time: 120 s, irradiation distance: 30 cm
6	MBP+MB9	
7	MBP+MB11	
8	MBP+MB12	
9	MBP+MB8	High intensity: 1.0 W, SP1 mode (repeatedly, irradiation 10 ms/stop 10ms), defocus, irradiation time: 3 s, irradiation distance: 2 cm
10	MBP+MB9	
11	MBP+MB11	
12	MBP+MB12	
13 (control)	DY+ MBP+MBB	No laser irradiation

Experimental Groups and Observation Terms

A summary of the experimental groups is shown in Table 2. The intensities of laser irradiation of experimental groups 1 to 4, 5 to 8, and 9 to 12 were set for low, medium (standard) with LLLT, and high intensity levels, respectively. In groups 1 to 12, each experimental bonding agent was applied to the exposed pulps after laser irradiation. However, in group 13 (control), DY was used as the direct pulp-capping agent without laser irradiation. Three postoperative observation terms were set: 3, 7, and 14 days. More specifically, rats were sacrificed 3, 7, and 14 days after direct pulp capping to prepare specimens for histopathological and immunohistochemical examination.

Specimen Preparation

The rats were anesthetized with diethyl ether (Wako Pure Chemical Industries; Osaka, Japan) and then deeply anesthetized by an intraperitoneal injection (40 mg/kgw) of 5% sodium pentobarbital (Nembutal, Dainippon Sumitomo Pharmaceutical; Osaka, Japan). After each rat was fixed on an operating board, the mouth was kept in an open position with a jaw prop. The teeth were cleaned with 3% hydrogen peroxide (H₂O₂ Oxydol, Sankyo; Tokyo, Japan), rinsed with a physiological saline solution, and then sterilized with diluted iodine tincture. Saucer-shaped cavities with a diameter

of approximately 0.5 mm were prepared on the mesial cusp of maxillary first and second molars on both sides by using a high-speed handpiece with a FG #440SS regular cut diamond point (Shofu; Kyoto, Japan) under a copious spray of distilled water. The pulps were then exposed with a #1/2 steel round bur (Shofu) attached to a low-speed handpiece under a copious spray of distilled water. The exposure had a diameter of approximately 0.2 mm. To stop the flow of blood from the exposed pulp and to produce sterility, 10% sodium hypochloride gel (NaClO gel, AD-Gel, Kuraray Medical) was applied to the cavity for 2 to 6 min. This was followed by alternate irrigation with 3% H₂O₂ and 6% NaClO solutions (Purelox, Oyalox; Tokyo, Japan) to remove dentin chips and NaClO gel. The cavity was then rinsed with a physiological saline solution, excess water was removed with sterilized small cotton pellets, and the cavity was blown dry with a gentle air stream.

In each experimental group, the surface of the exposed pulp was irradiated with the CO₂ laser apparatus according to each irradiation condition. After laser irradiation, MBP was applied to the cavity and left for 20 s, and then gently blown dry. Each experimental bonding agent was applied to the cavity including the surface of the exposed pulp as a direct pulp capping agent, blown dry, and then photopolymerized with a light-curing unit (Candelux, Morita; Tokyo, Japan) for 10 s. In the control group, MBP was applied to the cavity and left for 20 s, then dried by blowing air gently without laser irradiation, and the surface of the ex-

posed pulp was covered with DY. MBB was then applied to the cavity, blown dry gently, and photopolymerized for 10 s. After direct pulp-capping and bonding procedures, all the cavities were restored with resin composite, AP-X, and photopolymerized for 40 s.

Perfusion Fixation

The rats were sacrificed by an intraperitoneal injection of 5% pentobarbital sodium after each observation period. Each pulp was fixed by transcardial perfusion with a 4% paraformaldehyde phosphate buffer solution (pH 7.4). The maxillae containing experimental teeth were carefully removed and immersed in a 4% paraformaldehyde phosphate buffer solution at 4°C for 2 days.

Tissue Preparation and Serial Sectioning for Histopathological and Immunohistochemical Observation

We removed excess tissue from the maxillae and decalcified them with a 10% EDTA-2Na solution (pH 7.4) at room temperature for three to four weeks. After decalcification, AP-X was removed from the cavity and rinsed with running water for 24 h. The specimens were dehydrated in ascending grades of ethanol, dealcoholized by xylene, and then embedded in paraffin. Serial sections 5 to 6 μm in thickness were cut with a sliding microtome in a room with the temperature at 20 to 23°C and relative humidity between 50 and 60%. They were alternately stained with Mayer's Hematoxylin-Eosin staining to observe the pathological configuration, van Gieson staining to observe the formation of collagen fibers, connective tissue and hard tissue of dentin, Hucker-Conn staining to observe bacterial invasion, and modified NF Watanabe silver impregnation staining to observe reticulum fibers. As immunohistochemical staining, the sABC method on DMP1 staining was used to observe Dentin Matrix Protein 1 (DMP1) and the sABC method on TGF β 1 staining to observe Transforming Growth Factor β 1 (TGF β 1).

Observation Items and Evaluation Criteria

The stained sections were observed under the light microscope (Eclipse E1000, Lot #11545, Nikon; Tokyo Japan) and the following items were evaluated: pulp tissue disorganization, inflammatory cell infiltration,

reparative dentin formation, and bacterial penetration. The findings were evaluated according to the following criteria established by Medina III-Katoh:⁴⁰

A: Pulp tissue disorganization

1. Normal or almost normal tissue morphology (none).
2. Odontoblast layer disorganization, but the deep part of the pulp was normal (mild).
3. Loss of general tissue morphology (moderate).
4. Necrosis in the coronal one-third or more of the pulp (severe).

B: Inflammatory cell infiltration

1. Absence or presence of a few scattered inflammatory cells in the pulp (none).
2. Mild acute/chronic cell lesions (mild).
3. Moderate inflammatory cell lesions seen as abscesses or densely stained infiltrates of polymorphonuclear leucocytes, histiocytes and lymphocytes in one-third or more of the coronal pulp and/or the mid-pulp (moderate).
4. Pulp necrosis due to a severe degree of infection or lack of tissue in one half or more of the pulp (severe).

C: Reparative dentin formation

1. No dentin bridge formation (none).
2. Initial dentin bridge formation extending to not more than one-half of the exposure site (initial).
3. Partial/incomplete dentin bridge formation extending to more than one-half of the exposure site but not completely closing the exposure site (partial).
4. Complete dentin bridge formation (complete).

D: Bacterial penetration

1. Absence of stained bacterial profiles in any of the sections (none).
2. Presence of stained bacterial profiles along the coronal or apical walls of the cavity (mild).
3. Presence of stained bacterial profiles within the cut dentinal tubules or axial wall of the cavity (moderate).
4. Presence of stained bacterial profiles within the dental pulp (severe).

Immunohistochemical Staining

The sections were deparaffinized with xylene, dehydrated in ascending grades of ethanol, and then rinsed briefly with tap water and phosphate buffered saline

**Table 3** The diameters of exposed pulp area

Groups	After 3 days	After 7 days	After 14 days
1	0.413 [0.141], ^a (0.233-0.593)	0.336 [0.069], ^a (0.230-0.413)	0.333 [0.091], ^b (0.170-0.441)
2	0.296 [0.097], ^b (0.115-0.389)	0.235 [0.111], ^b (0.137-0.395)	0.355 [0.115], ^b (0.246-0.570)
3	0.234 [0.123], ^b (0.074-0.450)	0.221 [0.061], ^b (0.142-0.312)	0.299 [0.130], ^b (0.125-0.493)
4	0.196 [0.053], ^b (0.124-0.290)	0.196 [0.076], ^b (0.112-0.315)	0.263 [0.130], ^b (0.106-0.422)
5	0.189 [0.047], ^b (0.107-0.246)	0.211 [0.070], ^b (0.148-0.340)	0.290 [0.071], ^b (0.190-0.379)
6	0.279 [0.080], ^b (0.146-0.372)	0.144 [0.059], ^b (0.078-0.221)	0.357 [0.102], ^b (0.228-0.506)
7	0.207 [0.069], ^b (0.129-0.322)	0.149 [0.021], ^b (0.129-0.185)	0.269 [0.158], ^b (0.046-0.459)
8	0.249 [0.110], ^b (0.149-0.395)	0.217 [0.064], ^b (0.105-0.287)	0.231 [0.056], ^b (0.136-0.308)
9	0.185 [0.042], ^b (0.143-0.258)	0.171 [0.033], ^b (0.125-0.227)	0.253 [0.071], ^b (0.145-0.330)
10	0.204 [0.060], ^b (0.148-0.293)	0.158 [0.035], ^b (0.112-0.215)	0.339 [0.040], ^b (0.267-0.383)
11	0.192 [0.052], ^b (0.123-0.280)	0.147 [0.035], ^b (0.107-0.200)	0.240 [0.115], ^b (0.111-0.393)
12	0.231 [0.029], ^b (0.193-0.275)	0.258 [0.110], ^a (0.122-0.388)	0.279 [0.085], ^b (0.115-0.34)
13	0.246 [0.014], ^b (0.225-0.266)	0.295 [0.058], ^a (0.230-0.392)	0.307 [0.068], ^b (0.242-0.427)

[]: standard deviation, (minimum-maximum), unit: mm. Same superscript letters indicate no statistically significant differences for each postoperative observation term.

(pH 7.4). The sections were incubated with primary rabbit antibodies, such as anti-DMP1 antibody working dilution, 1:3000 for 12 h at 4°C Polyclonal (Lot #002FD, Takarabio; Siga, Japan) or anti-TGFβ1 antibody working dilution, 1:8000 for 12 h at 4°C anti-TGFβ1(V) (Lot #F2306, Cosmobio; Tokyo, Japan). They were immunochemically stained with Histofine SAB-PO(R) Kit (Lot #H0609, Nichirei Biosciences; Tokyo, Japan) employing the Avidin-biotin Horseradish Peroxidase Complex (sABC) method. The antibody localized antigen was then detected by peroxidase activation of 3,3-diaminobenzidine, DAB simple stain (DAB solution, Lot #H0610, Nichirei Biosciences). The sections were counterstained with Mayer's hematoxylin.

Measurement of the Diameter of the Exposed Pulp Area

The diameters of the exposed areas were measured with a stereomicroscope (Measuring Microscope MM-40, Lot #2104048, Nikon) and the widest dimension was recorded as the pulp exposure size of the specimen.

Statistical Analysis

The diameters of exposed pulp areas were statistically analyzed by one-way ANOVA and the Bonferroni post-hoc test with statistical software Microsoft Excel (Microsoft; Redmond, WA, USA) for differences among the experimental groups during each observation period at a significance level of 0.05.

The results of the histopathological evaluation were statistically analyzed by the Kruskal-Wallis H-test using Microsoft Excel for differences among the experimental groups during each observation period, and differences among the groups according to the observation period under each laser irradiation condition at a significance level of 0.05. Moreover, the correlation between inflammatory cell infiltration and bacterial invasion was investigated by the Kendall rank correlation using the statistical software SPSS (SPSS Japan; Tokyo, Japan) at a significance level of 0.05.

RESULTS

Diameters of Exposed Pulp Area

The mean diameters with standard deviations, and the maximum and the minimum values for the size of the exposed pulp areas of each group are shown in Table

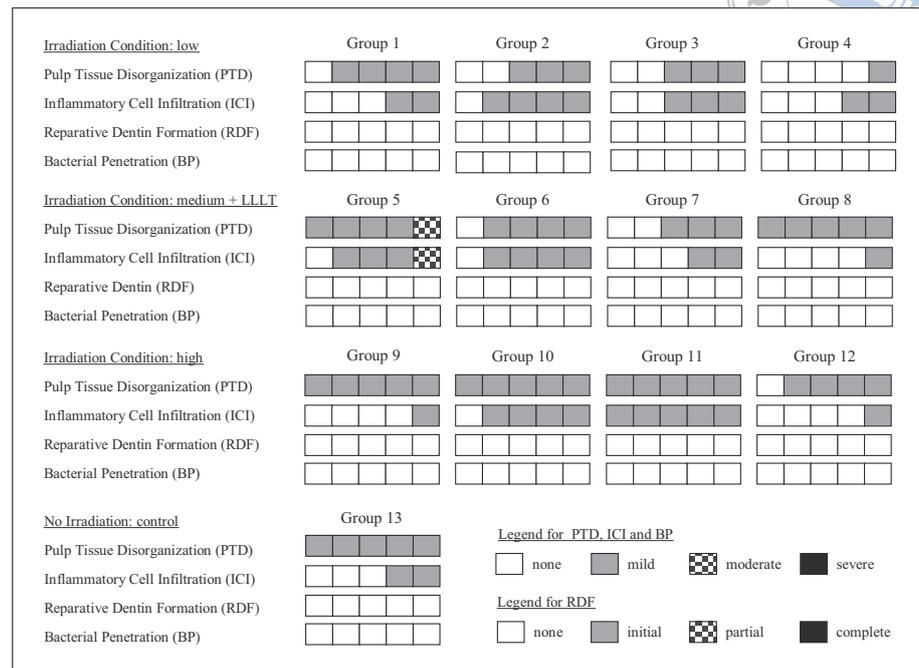


Fig 1 Results of histopathological and immunohistochemical evaluation (3 days).

3. The mean diameter of the pulp exposure of all specimens was 0.248 ± 0.105 mm, the maximum value of all pulp exposures was 0.593 mm and the minimum value was 0.046 mm.

There were significant differences among the pulp exposure sizes of the groups 3 and 7 days after treatment (one-way ANOVA, $p < 0.05$), although there was no significant difference among the pulp exposure sizes of the groups after 14 days (one-way ANOVA, $p > 0.05$). In the groups after 3 days, the size of exposed pulp area of group 1 was significantly larger than that of the other groups (Bonferroni test, $p < 0.05$). In the groups after 7 days, the size of the exposed pulp area of group 1 was significantly larger than that of the other groups except groups 12 and 13 (Bonferroni test, $p < 0.05$).

Histopathological and Immunohistochemical Findings

Fracture and or failing of the restorations were not observed in any of the specimens. A summary of the results of the histopathologic evaluation is shown in Figs 1 to 3. Representative histopathological and immunohistochemical images of all the groups are shown in Figs 4 through 8.

1) Histopathological and immunohistochemical findings after 3 days

The results of the Kruskal-Wallis H-test for each histopathological evaluation after 3 days revealed no significant difference among the experimental groups ($p > 0.05$). The eosinophilic heat denaturation layer and the protein coagulation layer were recognized in laser-irradiated areas under all irradiation conditions. The thickness of these layers had a tendency to increase as the intensity of laser irradiation increased.

One specimen in group 4 showed mild pulp tissue disorganization. Three specimens each from groups 2, 3, and 7 exhibited mild pulp tissue disorganization. Four specimens from each of the groups 1, 6, and 12 exhibited mild pulp tissue disorganization. In group 5, one specimen exhibited moderate pulp tissue disorganization, and the other specimens in total exhibited mild pulp tissue disorganization. In the other groups, five specimens exhibited mild pulp tissue disorganization.

Regardless of the irradiation condition, inflammatory cell infiltration was observed in most of the specimens. The inflammation area was enlarged as the intensity of laser irradiation increased. In groups 8, 9, and 12, four specimens in total showed no inflammatory cell infiltration and one specimen exhibited mild inflammatory cell infiltration. In the other groups, two to five specimens exhibited mild inflammatory cell infil-

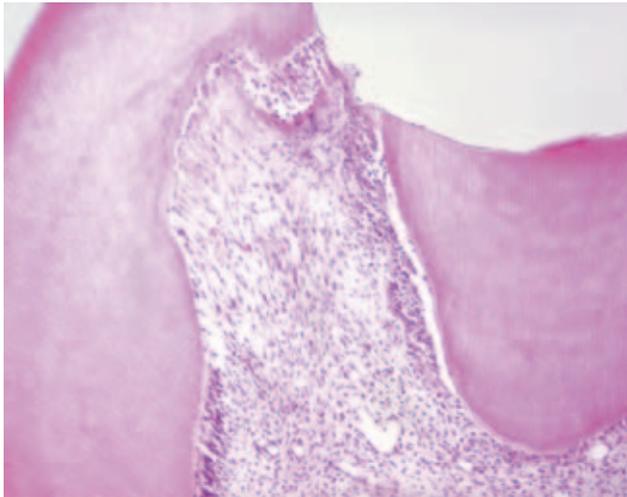


Fig 4 Histologic images of rat pulp irradiated using a high-intensity laser and capped with MB11 (group 11: 3 days) (H&E, 100X). The heat denaturation layer can be seen at the surface of the exposed pulp. The odontoblastic layer along the cavity dentin wall of the exposed site is disrupted and vacuolized. Deep pulp appears normal. There is no sign of either reparative dentin formation or bacterial penetration.

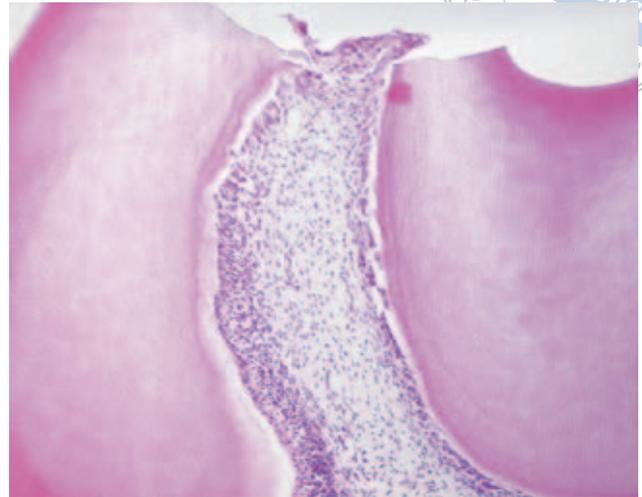


Fig 5 Histological images of rat pulp irradiated using a low-intensity laser and capped with MB9 (group 2: 7 days) (H&E, 100X). The heat denaturation layer can be seen at the laser-irradiated site. Recovery from disorganization in the odontoblastic layer can be recognized. Collagen fibers in the heat denaturation layer around the exposed pulp were stained purplish-red by modified NF Watanabe silver impregnation staining. Pulp tissue protruding into the cavity can be seen.

2) Histopathological and immunohistochemical findings after 7 days

The results of the Kruskal-Wallis H-test for each histopathological evaluation after 7 days revealed no significant difference among the experimental groups ($p > 0.05$).

In group 1, one specimen exhibited moderate pulp tissue disorganization, and the other specimens exhibited mild pulp tissue disorganization. Five specimens in group 8 exhibited mild pulp tissue disorganization. Four specimens in group 2 and the same number of specimens in group 5 showed mild pulp tissue disorganization. Two specimens from groups 10 and as many from group 11 demonstrated mild pulp tissue disorganization. One specimen in group 7 showed mild pulp tissue disorganization. In the other groups, 3 specimens in total exhibited mild pulp tissue disorganization.

In group 1, one specimen exhibited moderate inflammatory cell infiltration, and the rest exhibited mild inflammatory cell infiltration. Four specimens in group 8 showed mild inflammatory cell infiltration. Three specimens each in groups 2, 9, 11, and 12, exhibited mild inflammatory cell infiltration. One specimen in group 6 showed mild inflammatory cell infiltration. As

for the rest, a total of 2 specimens exhibited mild inflammatory cell infiltration.

Recovery from pulp tissue disorganization was observed in most of the specimens after 7 days. Neogenesis of blood vessels, odontoblasts, fibroblast-like cells, and predentin occurred in the area where collagen fiber was actively yielded. The thickness of the collagen fiber increased as the intensity of laser irradiation decreased. Recovery from pulp tissue disorganization was delayed when the intensity of laser irradiation increased. Eosinophilic heat denaturation layers and protein coagulation layers were recognized at laser-irradiated sites regardless of irradiation condition. The thickness of these layers had a tendency to increase as the intensity of laser irradiation increased. Calcium phosphate salt powder contained in MB8 was observed near the exposed pulp surface of one specimen. The pulp surrounded by the area supposedly penetrated by the laser beam was stained purplish red by modified NF Watanabe silver impregnation staining.

A tendency for the round type of cell infiltration to disappear was recognized under all irradiation conditions. Macrophages and monocytes were observed among collagen fibers and in the area around foreign objects. A positive reaction by TGF β 1 staining oc-

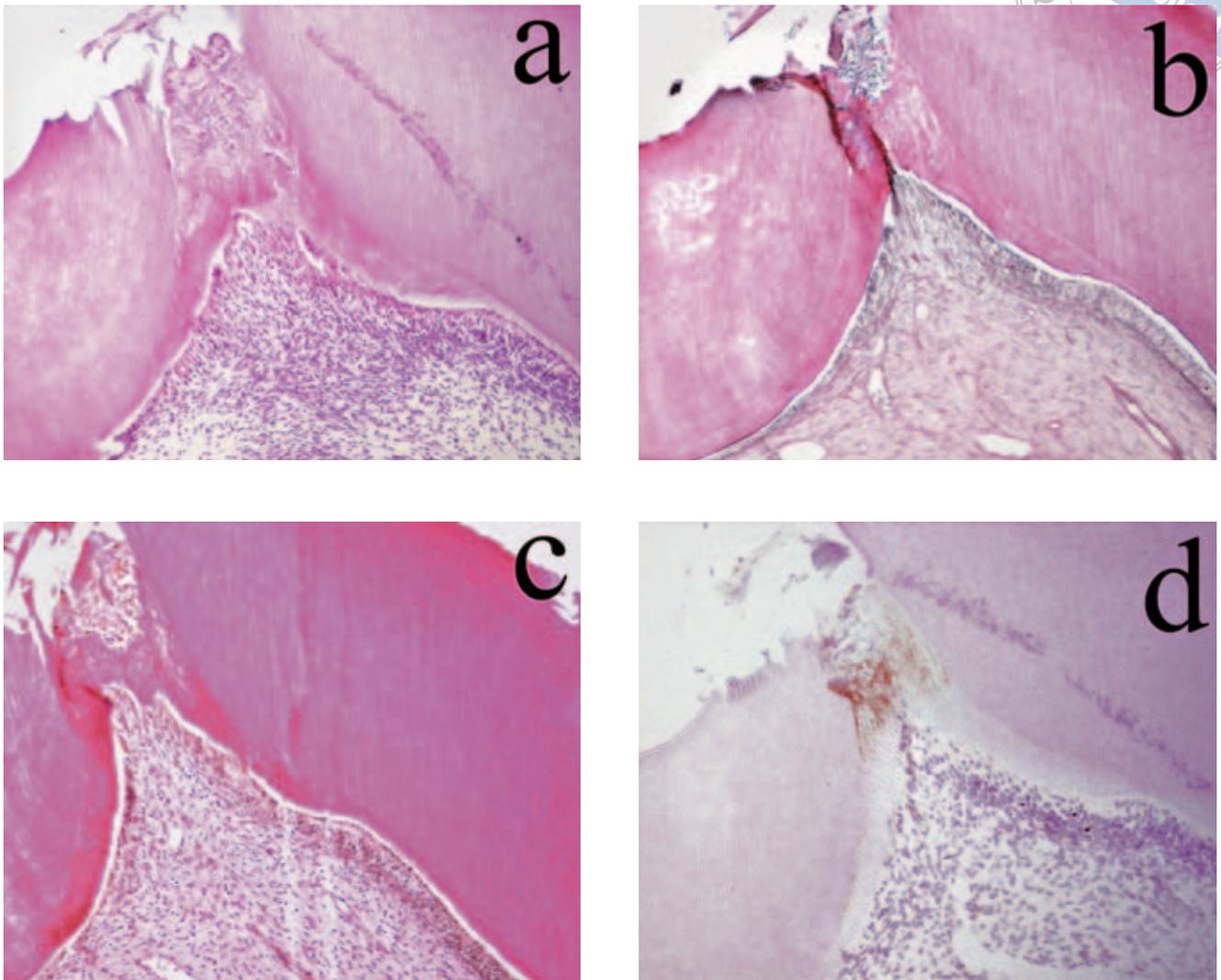


Fig 6 Histological images of rat pulp irradiated using a low-intensity laser and capped with MB12 (group 4: 14 days) (a: H&E, b: NF, c: van Gieson, d: DMP1, 100X). There is a complete dentin bridge that is composed of osteo-type dentin including odontoblast-like cells. A tubular-type dentin layer can be seen at the pulpal side of the dentin bridge. A positive reaction by DMP1 staining can be recognized in the dentin bridge.

curred in the area where round cell infiltration was observed.

Two specimens in group 6 showed thick reparative dentin formation. In group 12, moderate inflammatory cell infiltration in one specimen and severe inflammatory cell infiltration in another specimen were observed. One specimen in group 4 showed moderate reparative dentin formation. One specimen in group 1 showed mild reparative dentin formation. The other groups exhibited no reparative dentin formation.

Mild bacterial penetration was recognized in one specimen of group 2 (MB9) (low intensity of laser irra-

diation). However, there was no significant difference in the Kendall rank correlation between group 13 (control) and the other groups ($p > 0.05$).

3) Histopathological and immunohistochemical findings after 14 days

The results of the Kruskal-Wallis H-test for each histopathologic evaluation after 14 days revealed no significant difference among the experimental groups ($p > 0.05$).

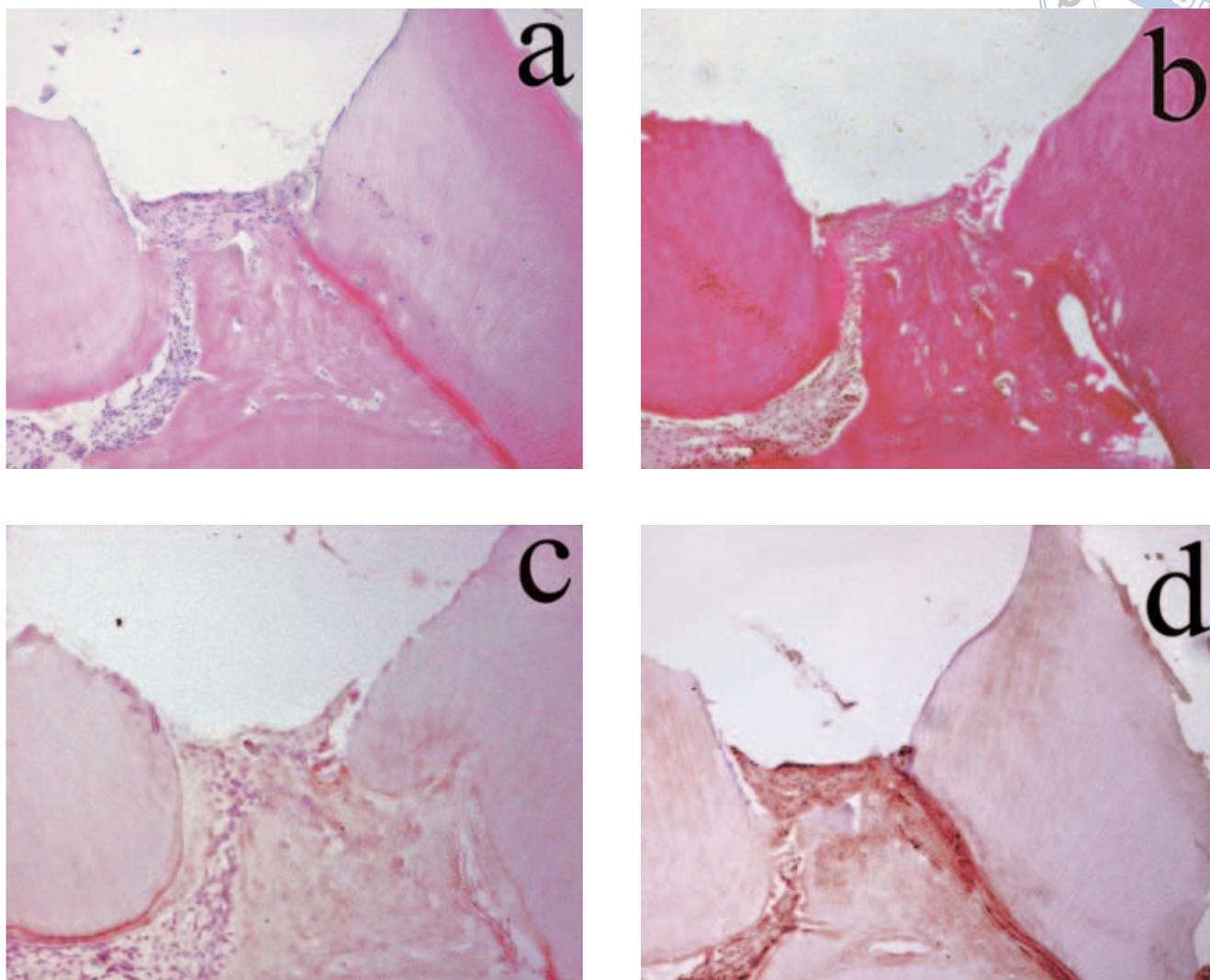


Fig 7 Histological images of rat pulp irradiated using medium-intensity with an LLLT laser and capped with MB9 (group 6: 14 days) (a: H&E, b: van Gieson, c: DMP1, d: TGF- β 1, \times 100). There is incomplete dentin bridge formation that is composed of very irregular osteo-type dentin and pulp tissue tunnel. A tubular-type dentin layer can be seen at the pulpal side of the dentin bridge. A positive reaction by DMP1 staining and that by TGF- β 1 staining can be recognized in the dentin bridge and in the pulp tissue, respectively.

In group 8, two specimens exhibited moderate pulp tissue disorganization, and the other specimens exhibited mild pulp tissue disorganization. Five specimens each in groups 5, 6, 7, 9, and 10 exhibited mild pulp tissue disorganization. Four specimens each in groups 3, 4, and 12 showed mild pulp tissue disorganization. Two specimens each in groups 1, 11, and 13 exhibited mild pulp tissue disorganization. One specimen in group 2 showed mild pulp tissue disorganization.

In group 8, two specimens exhibited moderate and one specimen mild inflammatory cell infiltration, and the rest showed no inflammatory cell infiltration. Five

specimens in group 10 showed mild inflammatory cell infiltration. Three specimens each in groups 1, 5, 9, and 12 exhibited mild inflammatory cell infiltration. Two specimens each in groups 3 and 13 showed mild inflammatory cell infiltration. One specimen each in groups 2, 4, and 11 showed mild inflammatory cell infiltration. The other groups exhibited no inflammatory cell infiltration.

Most of the specimens in the low-intensity irradiation group recovered almost normal tissue conditions. However, in the medium-intensity and high-intensity irradiation groups, most of the specimens had a tenden-

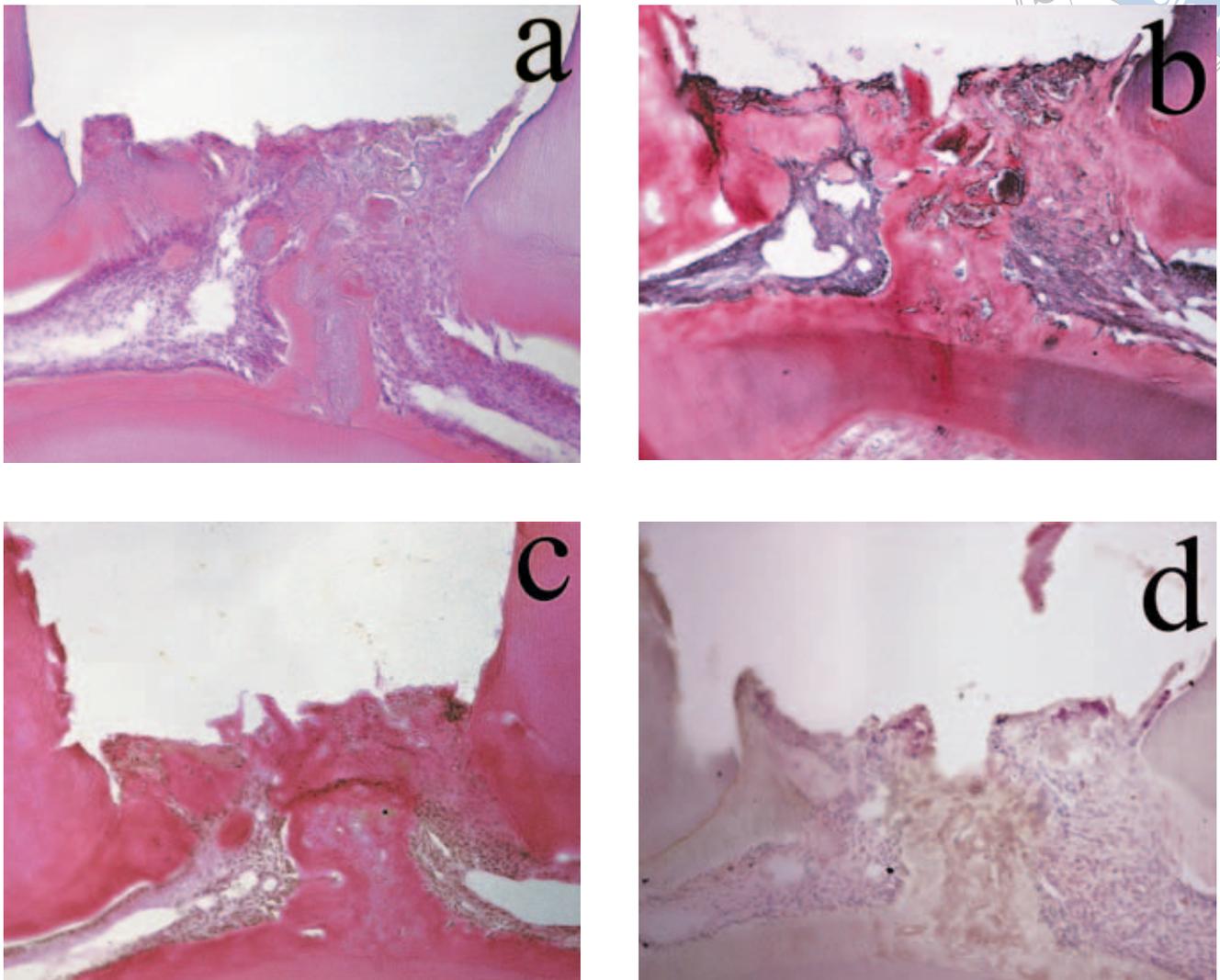


Fig 8 Histological images of rat pulp irradiated using a high-intensity laser and capped with MB11 (group 11: 14 days) (a: H&E, b: NF, c: van Gieson, d: DMP1, $\times 100$). There is almost a complete dentin bridge that is composed of irregular osteo-type dentin produced by calcification around the calcium phosphate particles. A positive reaction by DMP1 staining can be recognized especially in the remaining pulp tissue area of the dentin bridge.

cy to delay recovering, and some specimens showed almost the same results as the specimens of the low-intensity irradiation group did after 7 days. In the low-intensity irradiation group, remarkable hyperplasia of collagen fibers and fibroblast-like cells were observed around the wounded pulp surface where healing mechanisms were at work. Although the medium-intensity irradiation group showed almost similar histopathologic profiles of pulp tissue disorganization to those shown by the low intensity irradiation group, the former presented more aggressive hyperplasia of collagen fibers over a wider area than the latter. In the

high-intensity irradiation group, hyperplasia of collagen fibers covered a wider area than in the other irradiation condition groups.

Modified NF Watanabe silver impregnation staining revealed that the low-intensity irradiation group showed profiles rather similar to those of normal tissue, but that the medium-intensity and high-intensity irradiation group showed argyrophilic fibers underneath the dentin bridge.

Under all the irradiation conditions, round cell infiltration chiefly by lymphocytes and eosinophils was observed in almost all experimental groups. The spe-

cimens of the low-intensity irradiation group showed a tendency for hyperemia to disappear but for mild-round cell infiltration to remain.

On the other hand, in the specimens of the medium-intensity irradiation group, only a few cases of hyperemia were found. A few instances of vacuolar degeneration and mature granulocytes were demonstrated in some specimens of the high-intensity irradiation group. Macrophages were observed in the carbonization layer. A positive reaction by TGF β 1 staining was recognized in the round cell infiltration area. Recovery from inflammatory changes of the pulp were delayed when a higher-intensity laser irradiated the exposed pulp.

In the low-intensity irradiation group, the specimens to which MB9, 11, and 12 were applied showed almost complete dentin bridge formation with small tunnel-shaped defects, and their results were favorably comparable to those of the control group. Reparative dentin formation after 14 days had advanced compared to that observed after 7 days. Replacement of the odontoblast layer was observed underneath the reparative osteodentin. The surface of the exposed pulp was covered with irritative dentin protruding from the periphery of the pulpal wall. Some specimens showed dentin matrix formation among the collagen fibers. This finding was similar to that in the specimens after seven days. Pulp nodule formation was observed in a few specimens. These calcium deposits including the reparative dentin were found to be positive by van Gieson and DMP1 staining. The calcium deposits which occurred in the pulp tissue were a unique finding in the laser irradiation groups compared with the control. None of the specimens exhibited bacterial penetration 14 days postoperatively.

DISCUSSION

From the results of our previous studies,⁴¹⁻⁴⁴ the pulp response after direct pulp capping with laser irradiation seemed to be affected by laser irradiation levels and wavelengths, and showed a tendency to delay hard tissue formation. Therefore, we took the following matters into consideration in this study.

The Opelaser 03SII SP CO₂ laser device was used for irradiating exposed pulps of rat molars, because most of the energy of the CO₂ laser is absorbed within an area of 0.1 to 0.2 mm below the tissue surface and the thickness of the heat denaturation layer is less than 0.5 mm. Furthermore, the CO₂ laser has other merits, such as low caloric diffusion to the surrounding tissue,

effective control of hemorrhaging and exudation, and disinfection of the exposed pulp surface.

The intensities of the laser irradiation used in this study were decided upon from the results of our pilot study. The intensity of the laser irradiation used in our previous study⁵¹ was adopted as the medium intensity (standard) of the irradiation conditions, and the degree of intensity was decided within the range that permitted the formation of a carbonization layer on the surface of the rat exposed pulp. Laser irradiation with LLLT was added to only the medium intensity irradiation after the formation of a carbonization layer. It has been speculated that LLLT induces a photobioactive reaction (PAR), such as healing promotion during the inflammation stage, increase of fibroblasts and collagen production, and inhibition of excess granulation formation during the hyperplasia stage, regardless of laser type.⁵⁴ Consequently, we expected LLLT to control pulp inflammation and allow for earlier dentin bridge formation. In this study, laser irradiation with LLLT was only applied to the medium-intensity irradiation group for comparison with the other laser irradiation groups.

Takizawa et al⁴⁵ reported that pulp reaction after laser irradiation was related not only to energy consistency but also to output and exposure time of laser irradiation. In addition, Serebro et al⁴⁶ and Shoji et al⁴⁷ reported that pulp reaction to the CO₂ laser was more affected by irradiation time than output of irradiation. Therefore, the irradiation time was set as short as possible in this study. However, the results of this study showed that the thickness of the heat denaturation layer and protein coagulation layer tended to increase when the output of laser irradiation increased. Matsumoto et al⁴⁸ reported that the main cause of pulp irritation after laser irradiation was heat action, and the degree of pulp damage was dependent upon the characteristic of each laser apparatus, because heat absorption and reflection of the tooth surface was different from one type of laser apparatus to another. Accordingly, the degree of pulp irritation seems to increase in proportion to the heat storage capacity of dentin when a laser was irradiated. Selzer et al⁴⁹ and Zach et al⁵⁰ reported that when the pulp temperature increases more than 5°C pulp disorder results, and more than 11.1°C induces necrosis. There are other reports⁵¹⁻⁵² concerning thermal stimulus to pulp. In this study, the cooling function of the laser apparatus was used to protect pulp from heat caused by laser irradiation.

Jukić et al⁹ examined the effects of calcium hydroxide and CO₂ laser irradiation on the coronal portion of the removed vital pulp of a dog. They reported that upon histopathological examination 30 and 40 days

postoperatively, carbonization, necrosis, inflammatory cell infiltration, edema, and bleeding were observed, but that reparative dentin formation was hardly observed. The laser irradiation conditions used in this study were appropriate because restoration of the pulp tissue was recognized even in the tiny pulp cavity of the rat.

The results of histopathological examinations after three days showed a decrease of odontoblasts and pulp cells, irregular alignment of odontoblasts, and disappearance and vacuolar degeneration of predentin under all irradiation conditions. The thickness of heat denaturation layers increased as the intensity of laser irradiation was raised. The heat denaturation layer and carbonization layer served to protect the pulp. However, it was reported that excessive carbonization layer formation delayed the healing process.⁹ At 7 days postoperatively, the healing process of pulp tissue had already started: the neogenesis of odontoblasts and predentin and an active hyperplasia of collagen fiber were observed in most of the specimens, although delay in healing was recognized in a few specimens. Furthermore, most specimens under all irradiation conditions returned almost to normal 14 days postoperatively. Low-intensity laser irradiation was favorable for recovery of pulp tissue compared with high-intensity irradiation.

In this study, an experimentally developed bonding agent containing calcium phosphate salt was used as the direct pulp-capping agent. If the direct pulp-capping agent could not adhere to the wound surface due to uncontrolled hemorrhaging or exudation fluid, a space might be created between the pulp-capping agent and the wound surface. Therefore, it was speculated that a pulp tissue projection like a polyp may be produced by hyperplasia of pulp tissue into the space.^{19,29} Shirono et al⁴³ reported that the use of a focus laser beam in irradiating the exposed pulp promoted exudation or re-bleeding because of high energy consistency on the surface of pulp tissue. However, these actions by the focus beam were not observed in this study, since the CO₂ laser using a defocused mode was applied to the exposed pulp. Re-bleeding caused by MBP application, an exudation control deficiency after laser irradiation, and long laser irradiation time with the addition of LLLT action are problems which should be resolved in the future.

Inflammatory changes in the laser irradiation groups were more severe compared with those in the control. Inflammatory change of pulp tissue was observed 3 days postoperatively, and showed a decreasing tendency 7 days postoperatively. The experimental bond-

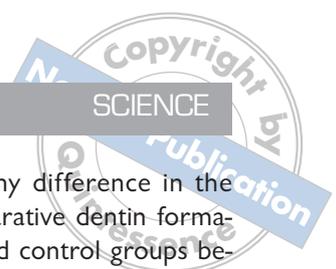
ing agent containing calcium phosphate showed good biocompatibility and might not cause irritation to the pulp. Various factors such as heat, vibration and pressure during cavity preparation, pulp injury at perforation, and hemostasis procedures were reported as the causes of pulp irritation after restoration by direct pulp capping.^{17,19,22,24,29,30} When a laser irradiated the exposed pulp before direct pulp capping, the heat action of laser irradiation was also considered responsible for delayed wound healing and delayed reparative dentin formation.

The present study found complete dentin bridge formation in part of the specimens to which low-intensity laser irradiation was applied, but incomplete dentin bridge formation in most of the specimens to which the medium- (standard) or high-intensity laser irradiation was applied, even 14 days postoperatively. It was speculated that delayed healing of pulp tissue irradiated with the high-intensity laser was due to damage by heat generation. The experimental teeth were cooled down by using the CO₂ laser apparatus with a coolant in this study. However, it was insufficient to remove the heat accumulation in dentin. Thus, the development of an efficient cooling system for laser irradiation is needed.

Bacterial penetration was observed on the cavity wall of only one specimen. The reason for this is presumably that plaque in the occlusal pit and fissure might mingle into the cavity during the restoration procedure, because a large bacterial mass was observed outside the cavity through the microscope. Disinfection by laser irradiation and good marginal sealing by the experimental bonding system used served to protect the pulp from bacterial penetration, which was limited to only one specimen in this study.

DMP1 and TGFβ1 were used for ensuring histopathological and immunohistochemical evaluation of the pulp. DMP1 and TGFβ1 antibodies were used as an index of reparative dentin formation and inflammatory change, respectively. DMP1 is used for confirming the formation of reparative dentin. This study used DMP1 as the index of reparative dentin formation and the area stained with DMP1 corresponded to the area stained with H&E and van Gieson.

TGFβ1 is a growth factor⁵³ for fibroblasts, promotes production of extracellular matrices, and produces granulation and arterIALIZATION simultaneously.⁵³⁻⁵⁷ However, it is said that TGFβ1 controls hyperplasia of cells such as macrophages and lymphocytes.⁵³ It is speculated that discharged TGFβ1 controls pulp cell hyperplasia and ALPase manifestation to contribute to the formation of reparative dentin after the pulp is damaged.⁵⁸ In this study, TGFβ1 showed a positive re-



action in the area of inflammation. Accordingly, it was suggested that TGF β 1 was related to the recovery of the wounded part of the pulp tissue. There was a significant difference between the pulp exposure sizes of the specimens observed 3 days postoperatively and those of the specimens observed 7 days postoperatively. However, this significant difference did not affect the histopathological results of this study.

Several studies reported that a dentin bridge was formed when a direct pulp-capping agent containing calcium phosphate salt was applied to the exposed pulp surface.^{16,24,29} On the other hand, no dentin bridge formation was reported in some other studies.^{19,22} Since the effect of laser irradiation on dentin bridge formation seems to be stronger than that of the experimental bonding system, the effect of the experimental bonding system used in this study was not clearly recognized. Good biocompatibility of calcium phosphate salt may contribute to the arrest of any inflammatory reaction. Calcium phosphate salt has been studied as a bone generative material. A mixture of calcium hydroxide powder and water or a physiological salt solution was applied to the exposed pulp as a direct pulp-capping material and to root canals as an antiseptic material. Calcification occurs under the condition of alkalinity produced by Ca and P ion from HAP and body fluid.¹⁷ Yoshida¹⁷ reported that crystalline needle-like structures were generated by the deposition of Ca and P around HAP and collagen fiber matrices. Additional hard tissue formation made by smaller particles of calcium phosphate started earlier than that made by larger particles of calcium phosphate, although the particle sizes of calcium phosphate did not differ in the pulpal reaction.²⁵ Furthermore, Katoh et al⁵⁹ speculated that Ca, P, and Mg ions in calcium phosphate salt might accelerate reparative dentin formation. Suzuki et al⁴⁴ reported that an experimental bonding agent containing calcium phosphate salt used for direct-pulp capping caused a less inflammatory response in the pulp and was effective in promoting reparative tubular-type dentin formation.

The mechanism of wound healing by laser irradiation has not been completely clarified. However, the thermal effect of laser irradiation is considered as a factor for recovery and calcification of the pulp. Several studies reported that mild damage to odontoblasts occurred immediately after laser irradiation, although they recovered over time, and finally reparative dentin was formed by regenerated odontoblasts.²⁻⁸ Another study reported that the photomechanical energy of a laser beam may contribute to hard tissue formation.⁶⁰

This study could not clarify any difference in the time, speed, and thickness in reparative dentin formation between the experimental and control groups because the heat effect of laser irradiation seemed to be too intense for the pulp. In MB12, a low-intensity irradiation group, two specimens showed complete dentin bridge formation and favorable results compared with the control. The thickness of the heat denaturation layer increased in proportion to the intensity of irradiation. Therefore, delay in pulp tissue healing after laser irradiation might be related to the thickness of the heat denaturation layer. From the results of this study, it was speculated that a thicker heat denaturation layer yielded by laser irradiation was attributable to thicker reparative dentin formation. However, the thick heat denaturation layer caused a delay in pulp tissue healing and might reduce the volume of the pulp cavity due to the formation of a considerable amount of reparative dentin. Accordingly, we recommend lower intensity laser irradiation than that used in this study.

CONCLUSIONS

Based on the results of this study, it was concluded that:

1. There were no significant differences in wound healing of exposed pulp among the three irradiation conditions (low-, medium- and high-intensity levels) of the CO₂ laser ($p > 0.05$), although low-intensity irradiation had a tendency to induce faster healing and better dentin bridge formation.
2. The thickness of irritative dentin formed by the side of the pulp cavity wall increased with the irradiation intensity of CO₂ laser.
3. There was no significant difference in the effect of direct pulp capping between the experimental bonding agents containing calcium phosphate and preparation of calcium hydroxide as the control DY ($p > 0.05$).
4. It was suggested that low-level irradiation with the CO₂ laser and direct pulp capping with experimentally developed bonding agents containing calcium phosphate were comparable to the preparation of calcium hydroxide DY.
5. CO₂ laser irradiation protected the surface of the exposed pulp through its functions of hemostasis, sterilization, and protein coagulation, and seemed to ensure the favorable effects of direct pulp capping.

ACKNOWLEDGMENTS

The authors thank the Kuraray Medical Co. for the experimental adhesive resins systems and other materials they generously provided. This study was supported by grants-in-aid for Scientific Research (B) (2) in 2003-2006, Project number: 15390578 from the Japan Society for the Promotion of Science.

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